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Polycation-dependent, Ca^{2+}-antagonized phosphorylation of calmodulin by casein kinase-2 and a spleen tyrosine protein kinase

Flavio Meggio, Anna Maria Brunati and Lorenzo A. Pinna

Istituto di Chimica Biologica dell 'Università and Centro delle Biomembrane del CNR, Via F. Marzolo 3, 35131 Padova, Italy

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Ten distinct protein kinases have been tested for their ability to phosphorylate calmodulin. Only casein kinase-2 and a spleen tyrosine protein kinase (TPK-III) proved effective, their phosphorylation efficiency being dramatically enhanced by histones and other polybasic peptides while being depressed by 50 μM Ca^{2+}. Phosphorylation by CK-2 takes place with a K_m of 12 μM calmodulin, leading to the incorporation of more than 1.5 mol P/mol substrate. Ser_{81} and Thr_{79} are among the residues affected. On the other hand, the two tyrosyl residues of calmodulin are both phosphorylated by TPK-III, Tyr_{99} being preferred over Tyr_{138}.

Calmodulin; Phosphorylation; Casein kinase; Tyrosine protein kinase

1. INTRODUCTION

Calmodulin is a small protein that mediates many of the physiological effects of Ca^{2+} (reviews [1,2]). Its primary structure is known [3] and its four domains capable of Ca^{2+} binding have also been localized (see [1]). Although several Ca^{2+}-dependent protein kinases [2] and the Ca^{2+}-dependent protein phosphatase, calcineurin [4], require calmodulin as a cofactor, no unambiguous indication that calmodulin itself could undergo phosphorylation was available. Recently, however, evidence has been provided supporting the concept that calmodulin undergoes phosphorylation under physiological conditions [5–7]. In particular, the occurrence in fibroblasts of calmodulin phosphorylated at both seryl and threonyl residues and the additional phosphorylation of calmodulin tyrosine residue(s) in RSV-transformed cells have been described [5]. These data prompted us to undertake an in vitro study aimed at identifying the protein kinase(s) potentially capable of affecting calmodulin. Moreover, considering that insulin-mediated phosphorylation of calmodulin requires the presence of histones [6], we have investigated the effect of polycations on the in vitro phosphorylation of calmodulin by the different kinases. The experiments described here actually support the view that polybasic peptides may play a crucial role in determining the phosphorylation of calmodulin by casein kinase-2 and by a tyrosine protein kinase (TPK-III) isolated from spleen. In contrast, eight other protein kinases, either Ser/Thr- or Tyr-specific, are unable to phosphorylate calmodulin appreciably either in the presence or absence of polycations.

Correspondence address: F. Meggio, Istituto di Chimica Biologica dell'Università and Centro delle Biomembrane del CNR, Via F. Marzolo 3, 35131 Padova, Italy

Abbreviations: A-MuLV, Abelson murine leukemia virus; CaM, calmodulin; CK, casein kinase; PK-C, protein kinase C; TPK, tyrosine protein kinase

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2. MATERIALS AND METHODS

Type 1 and 2 casein kinases (S and TS, respectively) were purified from rat liver [8]. Protein kinase-C, purified from pig brain [9], was kindly provided by Dr S. Ferrari. Phosphorylase kinase purified from rabbit skeletal muscle [10] was a gift from Professor P. Cohen (University of Dundee, Scotland). cAMP-dependent protein kinase was from Sigma. Four tyrosine protein kinases (TPK I, IIA, IIB, III) were partially purified from the particulate fraction of rat spleen by subjecting the three fractions previously isolated by DEAE-Sepharose column chromatography (I–III) [11] to affinity chromatography through a heparin-Sepharose column (1.5 × 4 cm) equilibrated with 25 mM Hepes, pH 7.0, containing 10% glycerol, 1 mM EDTA, 0.1% Nonidet P40, 10 mM 2-mercaptoethanol and 100 μM PMSF and eluted with a 0–0.5 M NaCl gradient in the same buffer. Heparin-Sepharose resolves fraction TPK-II into two subfractions, TPK-IIA and TPK-IIB eluted by 0.15 and 0.45 M NaCl, respectively. A preparation of the tyrosine protein kinase expressed by Abelson murine leukemia virus and free of other TPK activity was obtained from the cytosol of ABC-1 cells [12], and partially purified by DEAE-Sepharose chromatography as in [11]: a single peak of TPK activity was eluted with 0.75 M NaCl. Calmodulin was purified to homogeneity from calf brain essentially as in [13], with some modifications [14].

Phosphorylation experiments were routinely performed under optimal conditions for each protein kinase (detailed in [8,10,11,15]) by incubating calmodulin (at a final concentration of 40 μg/ml unless indicated otherwise) with 10 μM [γ-^{32}P] ATP (spec. act. 1.2 mCi/μmol), and 50 units of the single protein kinases. One unit is defined as the amount of enzyme transferring 1 pmol P/min into the model substrate protein (casein for CK-1 and CK-2, histones III A Sigma, for cAMP-dependent PK and PK-C, phosphorylase *b* for phosphorylase kinase and poly(Glu,Tyr) 4:1 for tyrosine protein kinases). The reaction was stopped by addition of 2% SDS, 1% 2-mercaptoethanol, 2 mM EDTA (final concentration). Samples were boiled and subjected to 11% polyacrylamide gel electrophoresis, in the presence of 0.1% SDS. Slabs were stained with Coomassie brilliant blue, dried and autoradiographed. In some experiments the band of calmodulin was excised and eluted by stirring the minced gel overnight in 50 mM Tris-HCl containing 1% SDS.

Analysis of ^{32}P-amino acids was performed after partial acid hydrolysis with 6 N HCl (3 h at 105°C) by high-voltage paper electrophoresis at either pH 3.4 or 1.9.

3. RESULTS

Calmodulin (150 μg/ml) was submitted to phosphorylation by comparable amounts of five different Ser/Thr-specific protein kinases (cAMP-dependent PK, phosphorylase kinase, protein kinase C, casein kinase-1 and -2), four spleen tyrosine protein kinases (TPK I, IIA, IIB, III) and the tyrosine protein kinase expressed by the A-MuLV oncogene, in either the presence or absence of polylysine. ^{32}P incorporation was evaluated by polyacrylamide gel electrophoresis, followed by autoradiography. As shown in fig.1 in the presence of polylysine a remarkable phosphorylation of calmodulin was promoted by two out of the ten protein kinases tested, namely CK-2 and TPK-III. A much less evident radiolabeling was promoted by TPK-IIB and phosphorylase kinase. In the absence of polylysine a slow phosphorylation of calmodulin could be detected only upon addition of TPK-III, and to a lesser extent of CK-2. The K_m value of calmodulin for CK-2 in the presence of polylysine was calculated to be 12 μM.

As shown in fig.2 several basic polypeptides can replace polylysine as inducers of calmodulin phosphorylation by CK-2, including polyarginine, polyornithine, histones and protamine. The peptide $(Arg)_4$-Ala-Gly-$(Arg)_4$ is still effective, though less than larger basic proteins, while neither the free amino acids lysine and arginine nor polyamines like spermine, which is a stimulator of CK-2 [16], increase the phosphorylation of calmodulin to any appreciable extent. Similar results were obtained using TPK-III as phosphorylating enzyme (not shown).

Calcium antagonizes the polybasic peptide stimulated phosphorylation of calmodulin by either CK-2 or TPK-III: as shown in table 1 at concentrations that nearly saturate calmodulin without affecting at all the intrinsic activities of either CK-2 or TPK-III, tested on casein and

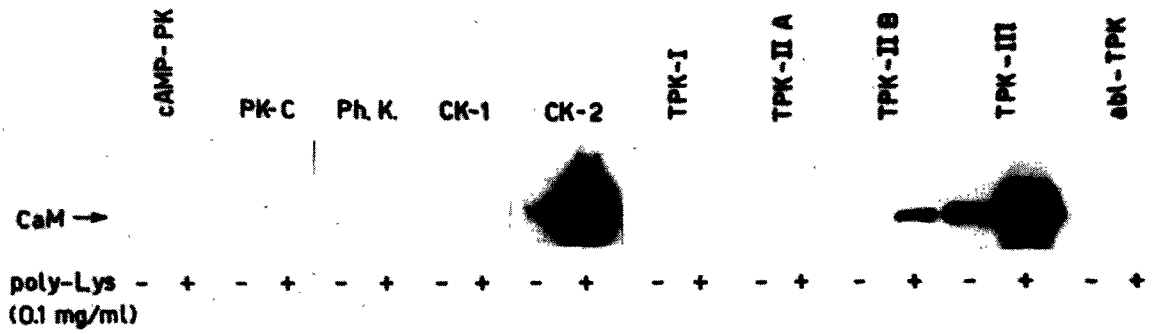

Fig.1. Specific phosphorylation of calmodulin by casein kinase-2 and a tyrosine protein kinase from spleen. Calmodulin (150 μg/ml) was subjected to phosphorylation by comparable amounts of five Ser/Thr protein kinases and five tyrosine protein kinases in either the presence or absence of polylysine (average molecular mass 37 kDa), as described in section 2. The protein kinases tested were: cAMP-dependent protein kinase, protein kinase-C (PK-C), phosphorylase kinase (Ph-K), casein kinases 1 and 2 (CK-1 and CK-2); four distinct tyrosine protein kinases isolated and partially purified from rat spleen (see section 2), indicated as TPK I, IIA, IIB and III, and the tyrosine protein kinase expressed by Abelson murine leukemia virus (abl-TPK). ^{32}P radiolabeling of calmodulin was evaluated by autoradiography (exposure time 24 h) after SDS-PAGE. The slabs relative to tyrosine protein kinases were treated for 2 h in 1 N NaOH at 56°C before autoradiography. Such treatment caused the disappearance of the radiolabeling induced by CK-2. The arrow denotes the position of calmodulin as detected by Coomassie staining. Only the gel sections including calmodulin are shown.

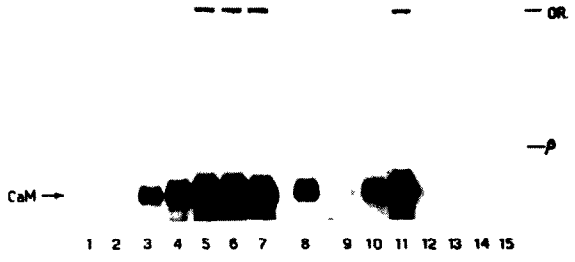

Fig.2. Stimulatory effect of different polycations on the phosphorylation of calmodulin by CK-2. Experimental conditions are described in section 2. Lanes 1 and 15 refer to controls with calmodulin alone and calmodulin plus CK-2 without any further addition. The added (poly)cations were: 2 mM lysine (lane 2); 20 μM polylysine, av. 5 kDa (lane 3); 80 μM polylysine av. 5 kDa (lane 4); 3 μM polylysine av. 37 kDa (lane 5); 3 μM polyornithine, av. 30 kDa (lane 6); 2.5 μM polyarginine av. 40 kDa (lane 7); 60 μM protamine (lane 8); 200 and 800 μM peptide Arg_4-Ala-Gly-Arg_4 (lanes 9 and 10, respectively); 27 μM histones, Sigma IIAS (lane 11); 2 mM putrescine (lane 12); 2 mM spermidine (lane 13) and 2 mM spermine (lane 14). CaM and β denote the position of calmodulin and of the autophosphorylatable β-subunit of CK-2.

poly(Glu,Tyr) 4:1, respectively, Ca^{2+} drastically reduces the phosphorylation of calmodulin. Mn^{2+} is less effective and Co^{2+} up to 500 μM is quite ineffective (not shown).

While, as expected, the radiolabeling by TPK-III is alkali-stable (fig.1) and is accounted for by phosphotyrosine (not shown), the radiolabeling by CK-2 vanishes upon alkali treatment, being accounted for by both phosphoserine and phosphothreonine. As shown in fig.3 the ratio between these two phosphoamino acids is variable with time: after a fast phosphorylation, ^{32}P-serine reaches a plateau, whereas ^{32}P-threonine is formed more slowly and goes on increasing even after 1 h incubation. At that time all ^{32}P incorporated into calmodulin approximates 1.2 mol/mol protein, but higher levels (1.5–1.8 mol P) could be reached by prolonging incubation and adding fresh CK-2. It should be noted that the Ser ^{32}P/Thr ^{32}P ratio is also greatly altered by addition of Ca^{2+}, resulting in inhibition of serine phosphorylation that is much more pronounced than that of threonine (see table 1).

Calmodulin contains two tyrosyl residues at positions 99 and 138 [3] which are included in distinct CNBr fragments (CNB1 and CNB3),

Table 1

Effect of Ca^{2+} on calmodulin phosphorylation by TPK-III and CK-2

Additions	^{32}P incorporated (mol/mol calmodulin)		
	TPK-III	CK-2	
		Ser ^{32}P	Thr ^{32}P
None	0.30	0.6	0.23
$CaCl_2$, 50 μM	0.11	0.21	0.21
$CaCl_2$, 250 μM	0.06	0.09	0.19

Incubation time was 20 min under the conditions described in section 2. Aliquots of calmodulin phosphorylated by CK-2 were analyzed for their phosphoserine and phosphothreonine content as described in the legend to fig.3

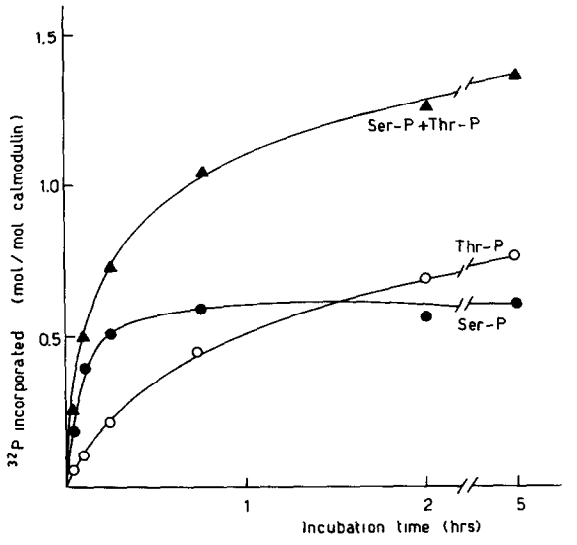

Fig.3. Time course of calmodulin phosphorylation by CK-2. Phosphorylation was performed in the presence of 3 μM polylysine (av. 37 kDa) for variable periods of time under conditions detailed in section 2. The radiolabeled bands of calmodulin were excised from the gel and their phosphorylation evaluated by liquid scintillation (▲). The relative amounts of ^{32}P-serine (●) and ^{32}P-threonine (○) were calculated by partial acid hydrolysis (6 N HCl, 4 h at 110°C) followed by high-voltage paper electrophoresis at pH 1.9. The radioactivities recovered in the spots of phosphoserine and phosphothreonine were corrected for hydrolytic losses of 50 and 20%, respectively.

readily separated by Sephadex G-50 gel filtration [16]. By applying such an approach to calmodulin phosphorylated by TPK-III it was ascertained that both residues are affected, Tyr_{99} being preferred over Tyr_{138}.

On the other hand, all of the radioactivity incorporated by CK-2 after either 10 or 120 min incubation is recovered in the large fragment CNB1 spanning between residues 77 and 124 and eluting first from Sephadex G-50. Its identification has been confirmed by amino acid analysis after DEAE-cellulose chromatography as in [16]. Four potential sites of phosphorylation are included in such a fragment; namely (i) Thr_{79}-Asp-Ser_{81}, (ii) Ser_{101}, (iii) Thr_{110} and (iv) Thr_{117}, the first and last representing good candidates for targets of CK-2 for having acidic clusters on their C-terminal side [17]. The actual phosphorylation of the first site at both its seryl and threonyl residues was established by tryptic subdigestion of the radiolabeled CNB1 fragment, generating three diversely sized peptides: the nonapeptide T2 including Thr_{79} and Ser_{81}, the hexadecapeptide T5B including Ser_{101} (together with Tyr_{99} affected by TPK-III) and the 18-residue-long C-terminal fragment including Thr_{110} and Thr_{117} [3]. As shown in fig.4A most of the radioactivity incorporated by 10 min incubation was recovered in a sharp peak of apparent M_r 1200, as judged by Sephadex G-50 gel filtration, containing both Ser ^{32}P and Thr ^{32}P (peak C), thus identifiable with the nonapeptide T2 including Ser_{71} and Thr_{81}. After prolonged incubation an additional tryptic fragment becomes radiolabeled exclusively at threonyl residue(s), which is less retarded by Sephadex G-50 (peak a, fig.4B). This peak partially overlaps that of the phosphotyrosine-containing peptide T2 (peak b) whose elution is intermediate between those of peaks a and c (fig.4B, dashed line). Collectively, these results show that among the five phosphorylatable residues of CNB1 Ser_{81} and to a lesser extent Thr_{79} are rapidly phosphorylated by CK-2, whereas a slower phosphorylation is undergone by Thr_{110} and/or Thr_{117}. Although we cannot discriminate at present between these two residues it is very likely that Thr_{117} rather than Thr_{110} is the target of CK-2 in virtue of the five acidic residues located on its C-terminal side which are known to act as specificity determinants for casein kinases of the second type [17].

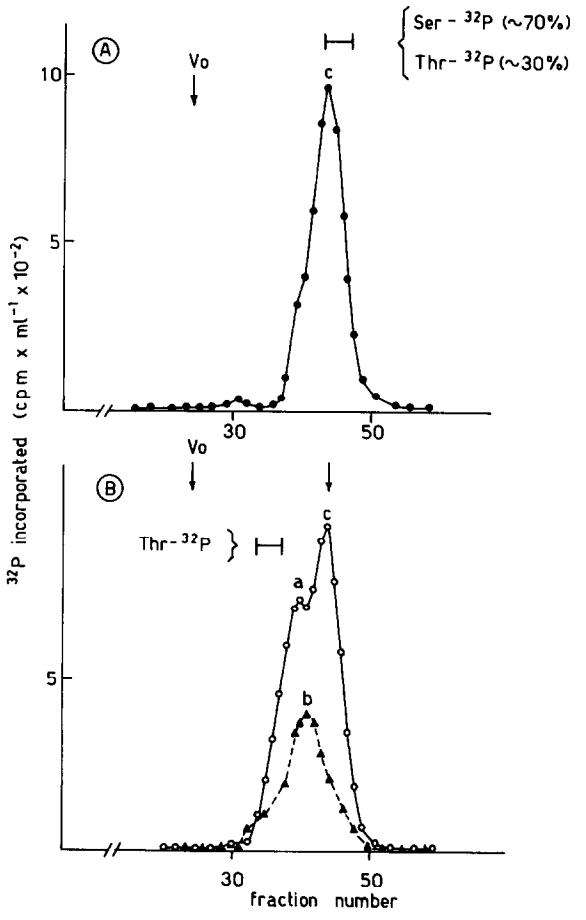

Fig.4. Isolation of radiolabeled tryptic fragments from calmodulin phosphorylated for either 10 min (A) or 2 h (B). ^{32}P-calmodulin phosphorylated by CK-2 as in fig.3 was first cleaved with CNBr. The fragment CNB1, accounting for the whole radioactivity incorporated, was isolated by Sephadex G-50 gel filtration followed by DEAE-cellulose chromatography, and subdigested with trypsin, as in [16]. The resulting peptides were subjected to gel filtration on a column (87 × 1.8 cm) of Sephadex G-50 equilibrated and eluted with 0.1 M NH_4HCO_3, pH 7.9. 4-ml fractions were collected and analyzed for radioactivity by counting 0.5-ml aliquots in a liquid scintillator. The Sephadex G-50 gel filtration profiles of tryptic fragments obtained from calmodulin phosphorylated by CK-2 for 10 min (●) and 2 h (○) are shown. In a parallel experiment calmodulin was radiolabeled by 2 h incubation with TPK-III and the tryptic fragment including the single phosphotyrosine of CNB1 was isolated by an identical procedure (▲). The arrow denotes the elution position of the decapeptide DAEYAARRRG (M_r 1270). Fractions were pooled and analyzed for their Ser ^{32}P and Thr ^{32}P content, as indicated.

4. DISCUSSION

This report describes the in vitro phosphorylation of calmodulin at either seryl and threonyl or tyrosyl residues by CK-2 and a tyrosine protein kinase from rat spleen (TPK-III), respectively. The phosphorylation process appears to be quite specific as eight other protein kinases tested proved nearly ineffective. Consequently type-2 casein kinases and tyrosine protein kinases related to TPK-III should be considered suitable candidates for carrying out the recently reported in vivo phosphorylation of calmodulin involving seryl, threonyl and tyrosyl residue(s). These data also corroborate the concept that tyrosine protein kinases display a remarkable substrate specificity similar to their Ser/Thr-specific counterparts: interestingly the capability of the cellular TPK-III to phosphorylate calmodulin is not shared by other cellular enzymes nor by the viral abl TPK, while pp60^{src} is also able to phosphorylate calmodulin [5]. Calmodulin appears to behave as an excellent substrate also for CK-2: its low K_m value (12 μM) and fast incorporation of more than 1 mol phosphate per mol protein are compatible with the physiological relevance of such a phosphorylation.

Interestingly, however, either histones or other polybasic peptides are required in order to ensure an efficient phosphorylation of calmodulin by CK-2. Since, on the other hand, the phosphorylation of casein, synthetic peptides and other substrates by CK-2, albeit enhanced by polycations, is not dependent on these effectors [18], it seems likely that in the case of calmodulin basic polypeptides act, at least in part, through a substrate-directed effect. This would be also consistent with the finding that histones greatly increase the phosphorylation of calmodulin by TPK-III, despite the fact that this protein kinase is totally insensitive to polycations when tested on other substrates (unpublished).

Calmodulin phosphorylation is largely prevented by Ca^{2+} concentrations which are expected nearly to saturate calmodulin without interfering with the activity of protein kinases. It is tempting therefore to assume a reciprocal antagonism between phosphorylation and Ca^{2+}-binding capacity of calmodulin. That actually phosphorylation could interfere with the activity of calmodulin as a mediator of calcium effects is also supported by

the localization of the phosphorylation sites: both Tyr_{99} and Tyr_{138} in fact, that are the targets of TPK-III, are within two of the four calcium-binding loops of calmodulin [1], while Ser_{81}, phosphorylated by CK-2, participates in the invariable sequence Ser-Glu-Glu-Glu which is suspected to represent the site responsible for calcium-dependent interaction with other proteins [3]: this makes especially notable the finding that the phosphorylation of Ser_{81} is more sensitive to low Ca^{2+} concentrations than that of threonyl residue(s) which are also affected by CK-2.

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